

## A LYTIC MOLECULE ACTIVE AGAINST A CILIATE DURING A TRANSMISSIBLE DISEASE OF *SIPUNCULUS NUDUS*<sup>1</sup>

FREDERIK B. BANG AND HYUN S. SHIN

*Station Biologique, Roscoff, France, and The Johns Hopkins University, Baltimore, Maryland*<sup>2</sup>

### ABSTRACT

A pox-like disease of *Sipunculus nudus* was transmitted experimentally and serially by scarifying the skin of normal specimens of *Sipunculus* and exposing them in an open dish of running seawater to spontaneously infected animals. Animals allowed to repenetrate the sand during the incubation period did not develop signs of infection.

A lysin which destroys ciliates (*Anophrys*), obtained from crab blood, appeared about 4-6 days after infection and persisted until death of the animal at 16-20 days from ulceration and secondary infection. The lysin was stabilized by mixing the cell-free fluid with 0.01 M EDTA, and full activity was recoverable in one peak on Sephadex-200 gel. The molecular weight was estimated as about 250,000 daltons.

### INTRODUCTION

Invertebrates have a variety of immune reactions (Shope and Maramorosch, 1975) and a greater variety of parasites (Kinne, 1980). Although invertebrates lack "antibody," they do have several serum substances that appear in response to disease. Among these substances is a lysin which appears in the blood of *Sipunculus nudus* (a marine coelomate) following the injection of a ciliate or in the course of a pox disease of the skin. This substance rapidly lyses the marine ciliate, *Anophrys*, thus providing a convenient *in vitro* test for activity. Since the reaction leads to complete lysis of the ciliate, it should eventually be examined in the context of evolution of the complement cascade in vertebrates.

Bang (1966) described the appearance and disappearance within the serum of *S. nudus* of a high titer of a lytic substance following the injection of large amounts of either *Anophrys* or certain gram-negative marine bacteria. At the same time, some animals used for other purposes in colonies of *S. nudus* maintained in aquaria at the Station Biologique developed a spontaneous skin disease characterized by pocks and ulcerations. This was followed by the development of the lysin. In subsequent summers at Roscoff, the disease was again noticed in the laboratory colonies, especially if the animals were kept in running seawater without the 6-in substrate of sand in which they normally burrow. High titers of lysin were present on each of three occasions when the disease occurred. This report presents data on: (i) production of the experimental disease following scarification; (ii) the continued association of the lysin with the disease; and (iii) the stabilization and beginning characterization of the lytic molecule.

Received 16 March 1981; accepted 27 April 1981.

Abbreviations used: R cells—presumptive regulatory cells; EDTA—ethylenediaminetetraacetate.

<sup>1</sup> Supported in part by NIH grant #5P 50HL-19157.

<sup>2</sup> Address for reprints.

## MATERIALS AND METHODS

To test for lysis, *Anophrys*-infected *Cancer pagurus* or *Carcinus maenas* crabs were maintained in the laboratory. Ten  $\mu$ l of crab blood containing approximately 200 *Anophrys* specimens were placed beside 5  $\mu$ l of test serum on a microscope slide. The drops were mixed rapidly with a needle. Five min after mixing, morphology and mobility of *Anophrys* were examined microscopically. Rounded organisms with indistinct edges were judged to be lysed. All tests were performed at room temperature.

The titer of the lysis was roughly quantitated as in Bang (1966) on the basis of rapidity and degree of lysis within 15 min. In the fractionation studies of the lysis (see below), the individual pools of 4 drops were saved and the positive ones retested within 2 h. The amounts of lysis varied about 10–20% from the first to the second test. This did not affect the titer as recorded.

To obtain a constant supply of high titer lysis for the characterization studies (see below), the skin of normal *Sipunculus* specimens was scarified, and the scarified animals were placed in direct contact with a spontaneously infected animal in running water without sand. For this purpose, a total of 35 animals, all of which had been kept in the aquarium away from the experimental animals, and which showed no spontaneous disease, were used. All had had access to sand and had remained buried under the sand. They were scarified with a sharp new needle by making light cross-hatched marks on their posterior ends, with the marks extending down into the corrugated skin of their sides. After this, the animals were placed in glass jars or plastic boxes with running water but minimal sand, and in direct exposure to infected animals. At the start, the infected animal was a spontaneously infected one. By the end of the summer, about five serial passages of the infection had taken place.

In the original study (Bang, 1966), the lysis was found to be highly heat-labile, and was destroyed by ether. When frozen at  $-20^{\circ}\text{C}$ , the lysis maintained some activity for 2–3 weeks, but at room temperature or at  $4^{\circ}\text{C}$  it was quite labile, so that characterization was difficult. In the present experiments, the addition of 0.01 M EDTA (ethylenediaminetetraacetate) considerably stabilized the activity and enabled us to carry out gel filtration studies. A column (0.5 cm inside diameter  $\times$  7 cm height) was packed with Sephadex G-200 beads and equilibrated in boiled filtered seawater containing 0.01 M EDTA at room temperature. One hundred  $\mu$ l of lysis was applied to the column and fractionated at a flow rate of 5 ml/h. The individual drops are recorded as fraction numbers. Four drops were collected to obtain a sufficient volume and assayed for lytic activity. Markers used were blue dextran (Pharmacia) and catalase (Sigma, molecular weight 232,000 daltons).

## RESULTS

Figure 1 shows the typical pock-like lesion of the early spontaneous disease. This, with time, progresses to ulceration. Small biflagellate protozoa (possible dinoflagellates) are often found in the lesions (Bang, 1966), but it is not clear whether they are primary or secondary invaders. Thus the etiological agent is undetermined.

Pock-like lesions were routinely produced along the lines of scarification in 13/14 animals that were scarified and left free of sand cover (Table I). The lesions appeared about 6–8 days after scarification. The lysis also was first detected in the blood at about the same time, but in several instances the lysis appeared a day or so before the lesions or vice-versa. Thus, a direct correlation between the two events was not established.

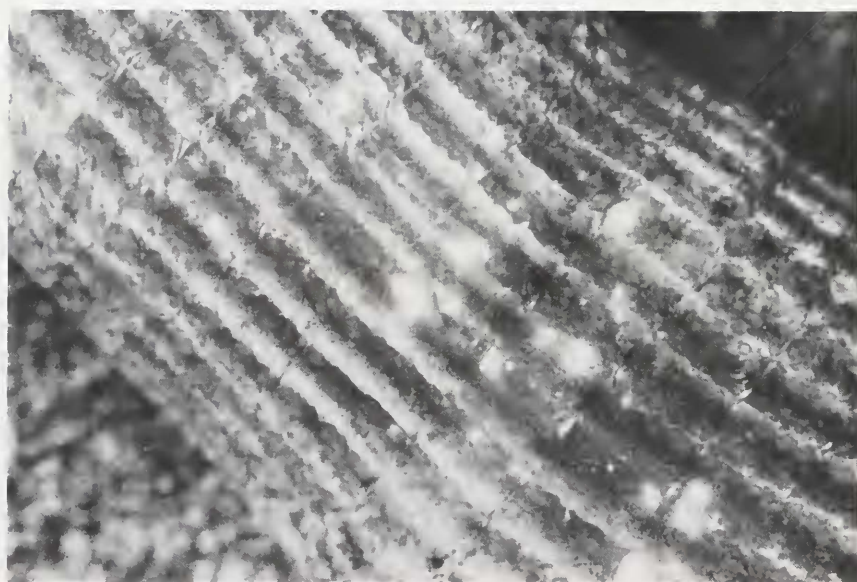


FIGURE 1a (above). Scattered spontaneous pox lesions on posterior region of *Sipunculus nudus*;  $\frac{1}{2} \times$  natural size. 1b (below) Spontaneous lesions of same animal;  $3 \times$  natural size. The lesions occur both between the muscular ridges and on top of them.

Neither lesions nor persistent lysin appeared in animals scarified, exposed, and allowed to go under the sand. In one final experiment (Table I, #12), seven animals were scarified, exposed to infected animals for 1, 2, or 3 days, and then allowed to burrow under the sand. In these, no significant high-titer lysin appeared, even though initial small lesions appeared as early as 3 days in the animals kept free of sand for 3 days. Lesions disappeared thereafter. The positive control animal, which was scarified and kept in open exposure, developed lesions and lysin. In another experiment, one animal scarified and kept out of sand for 17 days developed

TABLE I

Summary of experimental transmission of pox disease of *Sipunculus*. Injection was by intracoelomic injection of scrapings. *d* = days.

Expt.	No. of animals inoculated	Mode of maintenance	Mode of inoculation	Results
# 2	2	no sand	scarification	pox 6 d, lysin 8 d, 1 dead 22 d, 1 killed 18 d
# 3	1	no sand	scarification	lysin 4 d, no pox, dead 6 d
# 4	2	no sand	scarification	small lesions 6 d, lysin 12 d
# 5	2	sand	injection	no lesions, irregular lysin 2-9 d, recovery 10 d
# 6	2	no sand	injection	no lesion, poor lysin response
# 7	2	no sand	scarification	pox and lysin 6 d, killed 16 d
# 8	2	no sand	scarification	lysin 5-8 d, no lesions, recovery
	2	no sand	scarification	lysin and pox 5 d in one, other one not infected
# 9	2	no sand	scarification	pox and lysin 6 d
#10	4	sand	injection	no lesion, irregular lysin
#11	2	no sand	scarification	pox 7 d, lysin 9 d
#12	7	put in sand at intervals (see text), 1 kept out of sand	scarification	see text

sharply demarcated lesions and lysin by day 6. The animal then was allowed to go under the sand on the 17th day (Fig. 2, #27). Within 3 to 4 days, the external lesions disappeared, but the lysin persisted at high titer for 11 days after reestablishment of life under the sand, at which time the study was discontinued.

Injection of a suspension of scrapings from the skin lesion (Table I, experiments #5, #6, #10; total of 8 animals) caused temporary change in the lysin (possibly due to the contaminating bacteria), but no external lesions were apparent regardless of whether the animals were buried under the sand or exposed to running seawater.

The size of the specialized R cells on the circulating urn cell complex changed in several of the animals which developed skin lesions and lysin. These changes consisted of hypertrophy, elongation, and the "spontaneous" secretion of clear mucus apparently directly from these cells, and unassociated with the usual long tails of secretion seen when the urn cell complex is stimulated *in vitro* to secrete mucus tails. The regular production of a manifest pox disease following experimental exposure made it possible to follow the course of the serological reaction concomitantly with the overt disease. As Figure 2 shows, the lysin appeared 4-8 days after scarification and exposure, and was maintained thereafter in exposed animals.

As shown in Figure 3, lysin activity fractionated on a Sephadex G-200 column emerged as a single peak. Its molecular weight, as approximated by Andrews' method (Andrews, 1965) was 250,000 daltons. It is, however, possible that a more detailed analysis of this peak might show more than one component.

#### DISCUSSION

Although the infection occurs spontaneously in occasional animals left free of sand, and can now routinely be transferred from one animal to another, its etiology is not settled. A small protozoan, which is unidentified, appearing somewhat like



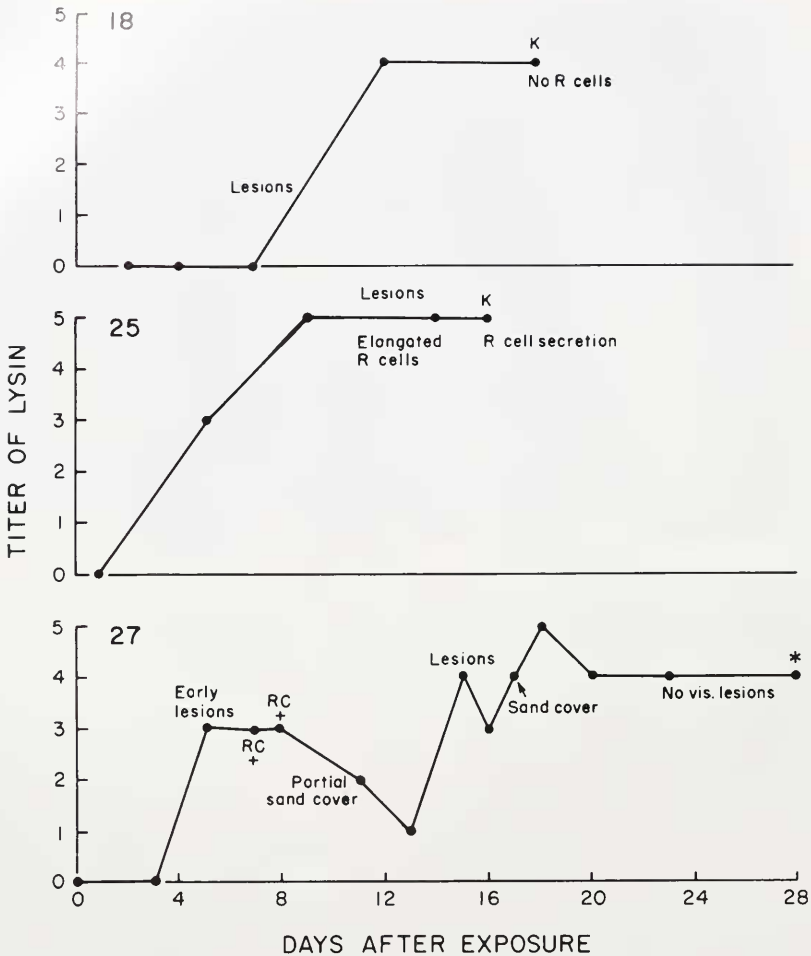


FIGURE 2. Course of disease symptoms and cellular reaction in typical pox disease induced by scarification and exposure. Each chart represents an individually numbered animal which had been scarified and exposed to a previously infected animal. The time of development of lesions was roughly correlated with the appearance of the lysin, but both varied by a few days. Animal #27 illustrates the decrease in lesions and lysin during early period under the sand, the reappearance of both under partial cover, and disappearance of lesions but persistence of lysin with complete cover. K = killed; RC = R cells positive for secretion; \* = cured, but experiment discontinued.

a euglenoid, was found frequently but not invariably in scrapings of the lesion, even when searched for repeatedly in early typical lesions. Thus the term "pox disease" refers only to the type of lesions, and the question of a virus etiology is untested.

The regular appearance of lytic activity in the course of this infection, the finding that the lysin can be stabilized in the presence of 0.01 M EDTA, and the likelihood that the activity is contained in one molecule, now raises the question of relationship of the lysin to the complement cascade in vertebrates. This cascade has two pathways. The alternate one unassociated with the antibody is presumably the more ancient phylogenetically. In the *S. nudus* lysin, the large molecule acts directly on *Anophrys* obtained from infected crab blood. When this lysin was tested on cultured ciliates of other species (unpublished), results were not as striking. The

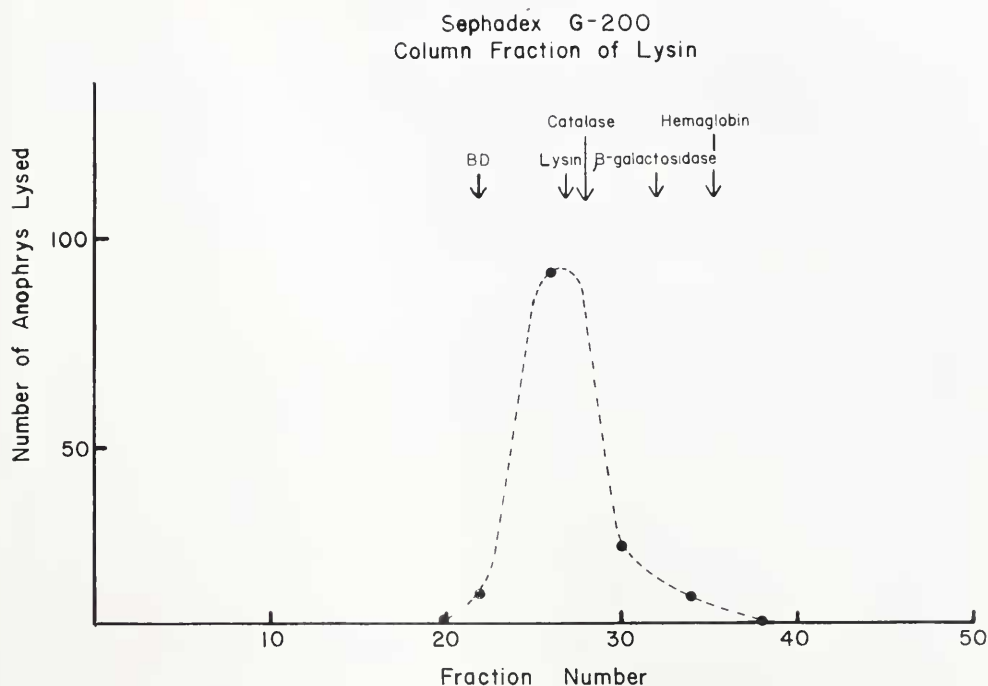


FIGURE 3. Fractionation of lysin from serum of spontaneously infected animals.

ciliates in crab blood may be "prepared" in some way for the *S. nudus* lysin, even though *Cancer* and *Carcinus* are unable to lyse them, since other crab species do at times produce a serum factor which clumps *Anophrys* and in rare cases lyses them (Bang, 1967).

The cellular source of the lytic molecule is unknown. It was originally shown that rapid but temporary release of the lysin occurred when *Sipunculus* blood was put in contact *in vitro* with foreign substances including crab blood containing *Anophrys* (Bang, 1966). This early lysin, however, may be separate from the persistent lysin here described. Possible sources are the clusters of secretory R cells within the autologous urn cell complexes (Bang and Bang, 1980), which swim freely in the *S. nudus* coelom. When high titers of lysin are present, the R cells hypertrophy and produce increased amounts of secretion (Bang and Bang, unpublished). The role of host amebocytes, or of the enigmatic plateletlike vesicles which enclose a fluid matrix, cannot be excluded.

### LITERATURE CITED

- ANDREWS, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96**: 595-606.
- BANG, B. G., AND F. B. BANG. 1980. The urn cell complex of *Sipunculus nudus*: a model for study of mucus-stimulating substances. *Biol. Bull.* **159**: 571-581.
- BANG, F. B. 1966. Serologic response in a marine worm, *Sipunculus nudus*. *J. Immunol.* **96**: 960-972.
- BANG, F. B. 1967. Serologic responses among invertebrates other than insects. *Fed. Proc.* **26**: 1680-1684.
- KINNE, O. 1980. *Diseases of marine animals. I. General aspects, Protozoa to Gastropoda*. John Wiley, Somerset, N. J. 460 pp.
- SHOPE, R. E., AND K. MARAMOROSCH, EDS. 1975. *Invertebrate immunity*. Academic Press, New York. 365 pp.